Comp. Biochem. Physiol. Vol. 93B, No. 2, pp. 239–246, 1989
Printed in Great Britain

0305-0491/89 $3.00 + 0.00
Pergamon Press plc

INTERACTION OF PARTIALLY-PURIFIED AMYLASES FROM LARVAL *ANAGASTA KUEHNIELLA* (LEPIDOPTERA: PYRALIDAE) WITH AMYLASE INHIBITORS FROM WHEAT*

J. E. BAKER

Stored-Product Insects Research and Development Laboratory, USDA, Agriculture Research Service, Savannah, GA 31403, USA

(*Received* 26 *July* 1988)

Abstract—1. Amylase from larvae of the Mediterranean flour moth (MFM), *Anagasta kuehniella* (Zeller), was partially purified by ammonium sulfate precipitation, glycogen complex formation, and chromatography on Sephadex G-100.

2. The amylase fraction was composed of two pairs of isozymes that were detected by polyacrylamide gel electrophoresis (PAGE). Each pair consisted of a major and a minor monomeric protein with molecular weights estimated with a non-dissociating PAGE technique to be about 51 kDa for the slower migrating pair and 56.5 kDa for the faster migrating pair.

3. Mediterranean flour-moth amylase had an alkaline pH optimum between pH 9.0–9.5, a K_m for soluble starch of 0.037%, and was unstable in acid (pH 4.5–5.0) conditions. Chloride ion did not activate the amylase nor cause a shift in K_m or pH optimum.

4. An aqueous extract of wheat acted as a non-competitive inhibitor ($K_i = 0.79\ \mu g/200\ \mu l$) of MFM amylase. Inhibition occurred through the range pH 5.5 to 9.5.

5. Mediterranean flour-moth amylase was not as sensitive as amylase from two granivorous coleopterans to two purified amylase inhibitors (*ca* 10 kDa) from wheat but was inhibited to a greater extent by partially purified wheat fractions that contained higher molecular weight albumins.

INTRODUCTION

Acidic amylases from granivorous coleopterans in the genera *Tenebrio*, *Tribolium*, *Oryzaephilus* and *Sitophilus* are sensitive to the complex of naturally-occurring amylase inhibitors present in wheat seeds (Silano *et al.*, 1975). These non-competitive inhibitors are proteins found in the albumin fraction of this cereal (Buonocore *et al.*, 1977) and have potential as biochemical resistance factors against several of these pest insects. The mechanism of inhibition of the enzyme is not completely understood. However, one of the major inhibitors from wheat forms a tightly-bound protein–protein complex with amylases from *Tenebrio molitor* L. ($K_i = 0.13$ nM) (Buonocore *et al.*, 1980) and *Sitophilus oryzae* (L.) ($K_i = 0.072$ nM) (Baker, 1988). Complex formation between inhibitor and enzyme may involve interacting tyrosine and tryptophan residues (Vittozzi *et al.*, 1987) as well as stabilization by ionic forces (Buonocore *et al.*, 1980; Powers and Culbertson, 1982). Since inhibition is affected by pH, it was of interest to determine sensitivity of amylases present in alkaline midguts of Lepidoptera (Day and Waterhouse, 1953; Wigglesworth, 1965) to these inhibitors and perhaps provide additional information on possible mechanisms of complex formation and inhibition. In this study, amylases from larvae of the Mediterranean flour moth (MFM), *Anagasta kuehniella* (Zeller), a granivorous pest species, were partially purified and their inhibition by whole wheat extracts as well as purified and partially-purified inhibitors was studied.

*Mention of a commercial or proprietary product does not constitute a recommendation by the U.S. Department of Agriculture.

MATERIALS AND METHODS

Insect cultures

A culture of *A. kuehniella* was obtained from John Riemann, USDA-ARS, Fargo, ND 58105. Larvae were reared at 28°C and 50–60% RH (16 hr photophase) on a 1:1 mixture of whole wheat flour and corn meal that contained 5% Brewers yeast.

Purification sequence

Preliminary studies indicated that amylase preparations from whole MFM larvae were identical to those of isolated midguts. Therefore mature larvae (14.3 g) were selected from stock cultures and homogenized with 60 ml 1% NaCl by using a Tekmar Tissuemizer. Crude homogenate was centrifuged 30 min at 5000 *g* (4°C). Supernatant was treated with ammonium sulfate at 4°C to obtain four fractions that precipitated between: (1) 0–0.5 M; (2) 0.5–1.5 M; (3) 1.5–2.5 M; and (4) 2.5–3.5 M. Precipitates were dissolved in 10 ml 50 mM glycine–NaOH pH 9.5 containing 20 mM NaCl and 0.1 mM $CaCl_2$ and dialyzed overnight at 4°C against this same buffer. Amylases in fractions 1 and 2 were further purified by glycogen precipitation (Loyter and Schramm, 1962; Baker and Woo, 1985).

Final purification of combined fractions 1 and 2 was by passage through a calibrated Sephadex G-100 column (1.6 × 92 cm) equilibrated in 50 mM Tris–chloride pH 7.5 containing 0.1 M NaCl and 0.1 mM $CaCl_2$. Flow rate was controlled at 0.5 ml/min with a peristaltic pump and 2 ml fractions were collected. Fractions were monitored for

protein by absorbance at 280 nm and for amylase activity. Calibration standards were bovine serum albumin, ovalbumin, chymotrypsinogen and ribonuclease A.

Purification of amylase was monitored by polyacrylamide gel electrophoresis (PAGE) using 7.5% gels and the anionic system of Davis (1964) on a vertical slab unit (Baker and Woo, 1985). pI values were determined using BioRad 3/10 ampholytes (Baker, 1982). Amylase zymograms were prepared according to Baker (1983) except the buffer used was 0.1 M glycine pH 9.5.

Assays

Amylase activity was monitored with the dinitrosalicylic acid (DNS) procedure as previously described (Baker, 1983; Baker and Woo, 1985). Maltose hydrate was used as standard. Protein was estimated with the procedure of Lowry *et al.* (1951) with bovine serum albumin as standard.

Molecular weight

Molecular weight of amylases was estimated under dissociating conditions by sodium dodecyl sulfate (SDS) polycrylamide gel electrophoresis on 12.5% gels (Baker and Woo, 1985) and under non-dissociating conditions by the method of Hedrick and Smith (1968) and Bryan (1977). The sample was also passed through a BioGel P-100 column (1.6 × 91 cm) equilibrated in 50 mM Tris–chloride containing 20 mM NaCl, 0.1 mM $CaCl_2$, and 0.02% sodium azide and calibrated with bovine serum albumin (66 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and ribonuclease A (13.7 kDa). Column flow rate was 0.3 ml/min and 2 ml fractions were collected. Tubes were monitored for protein (A_{280} nm) and for amylase activity.

Enzyme properties

Effect of pH on MFM amylase activity was determined by adding 20 μl of enzyme solution to 1 ml of 1% soluble starch in 0.1 M buffers containing 20 mM NaCl and 0.1 mM $CaCl_2$. Tris–chloride (pH 6.5–10), glycine–NaOH (pH 8.5–11), and borate (pH 8.5–11) buffers were used. Stock enzyme solution was diluted so that production of maltose equivalents at 30°C was linear with time through 30 min. Results are based on mean values of two separate tests with duplicates (corrected with starch blanks) at each pH.

The effect of chloride was tested by adding 20 μl of enzyme solution (diluted with 50 mM glycine pH 9.5 containing 0.5 mg/ml bovine serum albumin) to 1 ml of starch solution (8 concentrations from 0.067% to 0.5%) with or without 20 mM NaCl. Reaction was terminated after 10 min at 30°C by adding DNS reagent. Double reciprocal plots were prepared. Mean values are based on 3 separate experiments with different enzyme preparations and with duplicates (corrected for absorbance due to starch) at each starch concentration.

Preparation of wheat extracts

Crude extracts were prepared from soft red winter wheat ground to flour in a hammermill with 1/32″ openings. Three 1 g samples were each extracted with 5 ml 0.15 M NaCl for 1 hr at r.t. After centrifugation (30 min at 5000 ***g***), supernatants were heated at 70°C for 30 min, recentrifuged, and final supernatants passed through a 0.45 μm membrane filter.

Inhibition assay was as follows: MFM amylase was diluted so that 0.5 μg of partially-purified amylase protein was preincubated with various extract concentrations in a total of 200 μl of 50 mM glycine pH 9.5 containing 0.5 mg BSA/ml. Six extract concentrations (10–60 μl) taken from 200-fold dilutions (in glycine buffer with BSA) of each wheat extract were tested. After a 60 min preincubation at 30°C, 1 ml of 1% starch in glycine buffer was added for a 20 min reaction. Results are based on mean inhibition values of duplicates at each inhibitor concentration from each of the three extracts. One amylase inhibitor unit (AIU) is defined as the amount required to give 50% inhibition of 1 amylase unit (AU). One AU is that amount of enzyme that produces 1 mg maltose hydrate/min at 30°C.

Effect of pH on inhibition

The effect of pH on inhibition of MFM amylase by crude wheat extract was determined by two methods: (1) MFM amylase (0.5 μg) was preincubated with 60 μl of a 200-fold dilution of wheat extract in a total of 200 μl 0.1 M glycine adjusted to pH values of 7.5 to 11 by 0.5 unit intervals. At each pH, buffers were prepared with or without 20 mM NaCl and 0.1 mM $CaCl_2$. After 60 min at 30°C, 1 ml of 1% starch prepared in these same buffers was added for a 20 min reaction; and (2) MFM amylase (0.5 μg) was preincubated (with or without inhibitor) in a total of 200 μl 0.1 M acetate (pH 4.5–6.0); phosphate (pH 6.0–7.5); Tris (pH 7.5–8.5); or glycine (pH 8.5–10.5) buffer. Residual amylase activity was determined by adding 1 ml of 1% starch in 0.1 M glycine pH 9.0 for a 20 min reaction.

Purified inhibitors

Inhibitor-0.12 and inhibitor-0.31 were purified from wheat as previously described (Baker, 1988; 1989). Two additional fractions (P-100-1 and P-100-2) were from an inhibitor sample that was eluted from a DEAE–Sephacel column (tubes 70–90, Fig. 1, Baker, 1988) and subsequently separated by passing through a BioGel P-100 column (Baker and Woo, unpublished). Each inhibitor fraction was tested by preincubating 0.5 μg of MFM amylase with 6 inhibitor concentrations from 0.1 to 0.6 μg in 200 μl 50 mM glycine pH 9.5 with 0.5 mg/ml BSA for 60 min at 30°C. Residual amylase activity was measured as above.

RESULTS

Purification of MFM amylase

MFM amylase was purified 270-fold based on activity in the original larval homogenate and final activity in the sample eluting from the Sephadex column (Table 1). Most amylase activity in the crude homogenate precipitated with 0–0.5 and 0.5–1.5 M ammonium sulfate. Glycogen precipitates of these two samples were nearly identical in specific activity as well as protein composition based on PAGE. They were therefore combined prior to gel filtration on Sephadex G-100 (Fig. 1A). Amylase activity was associated with a small protein peak eluting from this column between tubes 60 and 78. This small peak eluted later than the lowest molecular weight standard protein (ribonuclease A, 13.7 kDa) and had an apparent molecular weight of 6.5 kDa by extrapolation of the plot of log mol. wt vs. K_{av}. Final specific activity of this sample was 94.4 AU/mg protein.

Two pairs of protein-stained bands were visible following PAGE analysis of the Sephadex G-100 sample (Fig. 1B). Each pair consisted of a major and a minor protein band. Major bands occurred at Rm 0.29 (band 1) and 0.57 (band 3) while minor bands occurred at Rm 0.33 (band 2) and 0.59 (band 4). All four bands (isozymes) were active in the starch zymogram. An additional minor protein-stained band was visible with heavier sample loadings at Rm 0.44. This band was weakly active in the zymogram. All amylase bands could be detected in individual midguts ($N = 40$) subjected to PAGE and zymogram analysis. This strain of *A. kuehniella* appears to be monomorphic for isoamylase composition.

Table 1. Purification of amylase from larvae of the Mediterranean flour moth, *Anagasta kuehniella*

Sample	Protein (mg)	Amylase* (units)	Sp. act.†	Recovery‡ (%)	Fold purification§
Larval homogenate	989	339.8	0.35	100	1
0.0–0.5 M	30	136.9	4.50	40.3	12.8
0.5–1.5 M	111	154.9	1.39	45.6	3.9
1.5–2.5 M	201	7.0	0.03	2.1	—
2.5–3.5 M	228	5.5	0.02	1.6	—
Gly ppt of 0–0.5 M	2.85	113.7	39.9	83‖	114
Gly ppt of 0.5–1.5 M	4.20	173.7	41.3	112‖	118
Sephadex G-100	0.55	51.9	94.4	15.3	270

*One amylase unit (AU) = 1 mg maltose hydrate/min at 30°C.
†AU/mg protein.
‡Based on total amylase units in original homogenate.
§Based on specific activity in original homogenate.
‖Based on amylase units in corresponding ammonium sulfate precipitate. These two samples were combined prior to Sephadex G-100 chromatography.

Two protein-stained bands could be detected in these partially-purified Sepadex G-100 samples following isoelectric focusing in 3/10 ampholytes. Mean pI values calculated from 2 runs were pH 4.2 (major band) and pH 3.5 (minor band). Amylase zymogram activity was found in rather broad zones, with the major band centered at about pH 4.0 and a minor band at about pH 3.5. Because of the heterogeneous nature of the protein sample, it was not possible to correlate protein bands observed by PAGE with those focused on gels containing ampholytes.

Molecular weight

MFM amylase gave a single protein-stained band following SDS–PAGE (Fig. 2). From a plot of log mol. wt against relative mobility, molecular weight of the band was estimated to be 53 kDa. Molecular weight estimates of each of the four isozymes were obtained by using the non-dissociating PAGE technique (Fig. 3). Calculations based on slopes of plots of relative mobilities at gel concentrations of 5–9% against molecular weights of protein standards gave the following estimates: band 1–51.4 kDa; band 2–49.7 kDa; band 3–56.5 kDA; and band 4–56.5 kDa. SDS–PAGE and PAGE results indicate that the isozymes are apparently monomeric proteins.

Similar to elution from Sephadex G-100, MFM amylase was severely retarded on BioGel P-100 and eluted far outside the range of the low molecular weight protein standards.

Effect of pH

MFM amylase was most active in the alkaline pH region (Fig. 4). Optimum activity occurred between pH 9 and 10 with maximum activity at about pH 9.5 in both the Tris and glycine buffers. Activity was slightly higher in Tris compared to glycine buffer whereas activity was much lower and shifted to a more alkaline optimum in the borate buffer system.

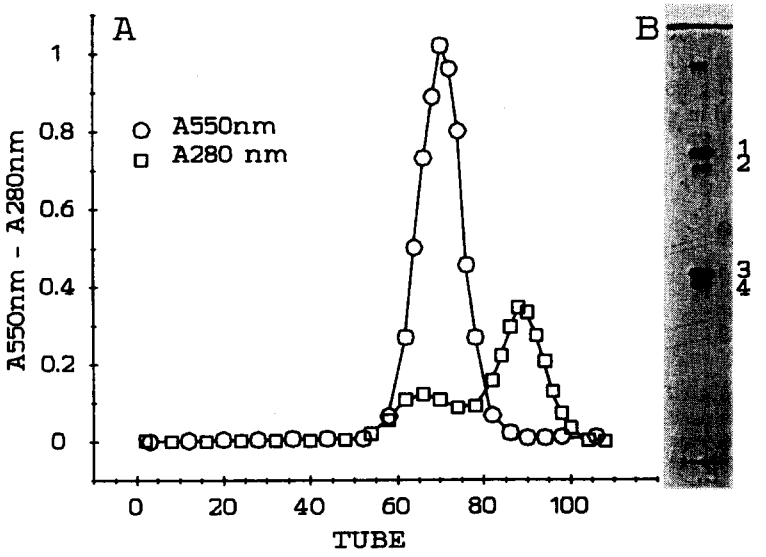

Fig. 1. (A) Elution pattern of MFM amylase through a Sephadex G-100 column (1.6 × 92 cm) equilibrated in 50 mM Tris–chloride pH 7.5 containing 0.1 M NaCl and 0.1 mM $CaCl_2$. Flow rate was 0.5 ml/min and 2 ml fractions were collected. Fractions were monitored for protein (A_{280}) and amylase activity (A_{550}). Tubes 62–76 were combined and concentrated. (B) PAGE analysis (7.5% gels) of MFM amylase (20 μg protein) from Sephadex G-100. Major proteins were located at Rm 0.29 (band 1) and 0.57 (band 3). Minor proteins were located at Rm 0.33 (band 2) and 0.59 (band 4). All four proteins were active in starch zymograms.

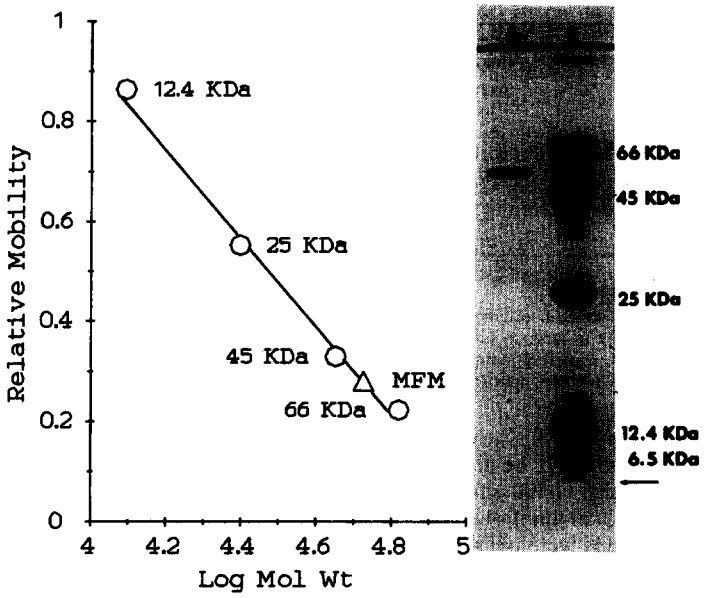

Fig. 2. SDS–PAGE analysis of MFM amylase showing plot of log molecular weight of protein standards against relative mobility on 12.5% gels. Inset: A single protein band was observed when MFM amylase (40 μg) was analyzed. Lane A—MFM amylase; Lane B—Protein standards: Bovine serum albumin (66 kDa), ovalbumin (45 kDa), chymotrypsinogen (25 kDa), cytochrome *c* (12.4 kDa), and aprotinin (6.5 kDa).

Effect of chloride

Chloride ion had no significant effect on V_{max} of MFM amylase or on its K_m for soluble starch (Fig. 5). Mean K_m values for starch were $0.037 \pm 0.004\%$ without chloride and $0.038 \pm 0.005\%$ in the presence of 20 mM chloride. In addition, the presence of chloride ion did not result in a shift in pH optimum of the enzyme (see below).

Inhibition of MFM amylase by wheat extract

To obtain a suitable range of inhibition of 0.5 μg MFM amylase in the 200 μl preincubation mixture, each wheat extract (1 g flour/5 ml 0.15 M NaCl) had to be diluted 200-fold in glycine buffer. The effect of concentration of these diluted samples on amylase activity is shown in Fig. 6(A). The following calculations were based on these data: (1) 1 AIU was equivalent to 27.8 μg soluble flour protein or 1.7 mg wheat flour; and (2) 1 g wheat flour contains 588 AIU against MFM amylase. A Dixon plot of these data, where reciprocal velocity (1/AU) is plotted against inhibitor concentration, was linear (Fig. 6B). *X* intercept of this plot gives the dissociation constant (K_i) of non-competitive inhibitors which was calculated to be 0.79 μg/200 μl. MFM amylase could not be completely inhibited by wheat extract under these preincubation conditions, even when undiluted crude extract was tested.

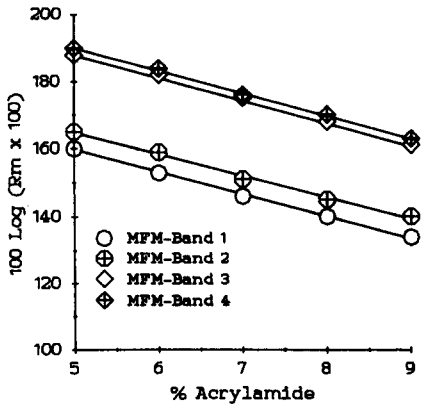

Fig. 3. Plots of log mobility against gel concentration during PAGE for the four isozymes that compose the MFM amylase sample. Slopes of these lines were used to estimate molecular weights. Calculated values were: band 1—51 kDa; band 2—49.7 kDa; band 3—56.5 kDa and band 4—56.5 kDa.

Effect of pH on inhibition

Wheat extract inhibited MFM amylase through the entire pH range of the glycine buffer (Fig. 7).

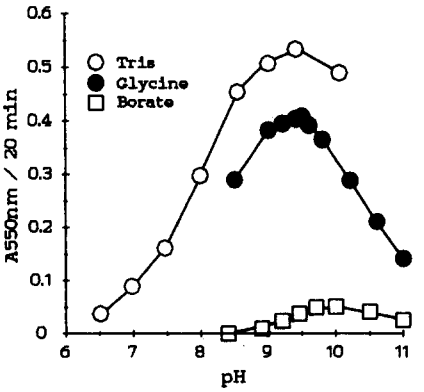

Fig. 4. Effect of pH on hydrolysis of soluble starch by MFM amylase. Values are means of two tests with duplicates at each pH.

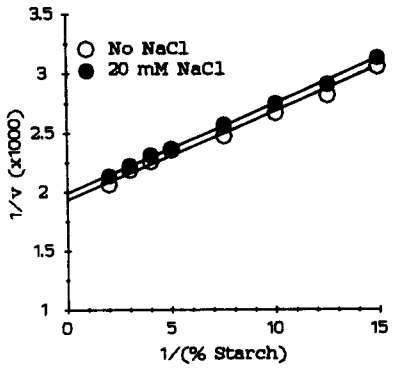

Fig. 5. Double reciprocal plots of effect of starch concentration (%) on activity (mg maltose/10 min) of MFM amylase in the presence of 20 mM NaCl. Data are means of 3 experiments with duplicates at each starch concentration. Intercepts were obtained with a linear program analysis. K_m values were 0.037% with no chloride present and 0.038% with 20 mM NaCl.

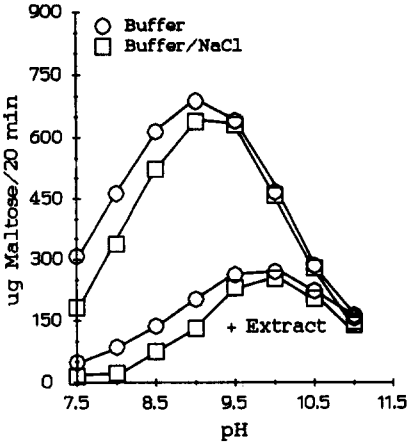

Fig. 7. Effect of pH on inhibition of MFM amylase by wheat extract. Buffers (50 mM glycine) were prepared with and without 20 mM NaCl. Residual amylase activity was measured at the preincubation pH.

Inhibition based on percentage of control was highest (84%) at pH 7.5 and decreased steadily as pH increased; i.e. inhibition was 71% at pH 9.0, 42% at pH 10, and 4% at pH 11. Chloride ion had no significant effect on percent inhibition. There was a slight shift in amylase activity to the alkaline side in the presence of wheat extract.

The effect of preincubation of MFM amylase (control) and MFM amylase plus wheat extract in a wide range of pH values on residual amylase activity assayed at pH 9.0 is shown in Fig. 8. Although preincubation in acidic conditions (pH 4.5–5.0) resulted in a loss in enzyme activity, inhibition by the extract was still apparent at these pH values. Interestingly, preincubation of MFM amylase at pH 10 and pH 10.5 slightly activated the enzyme, whereas inhibition decreased in these alkaline conditions. When these data are expressed in terms of percentage inhibition (Fig. 9), it is apparent that the inhibitory components of the wheat extract interact with the enzymes through a rather broad pH range, approximately pH 5.5 to pH 9.5. Inhibition decreases abruptly outside this pH range.

Effect of purified inhibitors

MFM amylase (0.5 μg/200 μl) was not inhibited by inhibitor-0.12 at concentrations up to 1.0 μg in the preincubation mixture. Inhibitor-0.31 was only slightly inhibitory under these conditions (Fig. 10A). Fractions P-100-1 and P-100-2 were much more inhibitory on a weight basis. Reciprocal plots of these latter two fractions are given in Fig. 10(B). K_i for P-100-2 was estimated to be 0.98 μg. The reciprocal plot of P-100-1 was non-linear under these conditions; however, extrapolation of this data to the X axis gave an estimated K_i of 0.49 μg which is a measure of the higher sensitivity of MFM amylase to components in this inhibitor fraction.

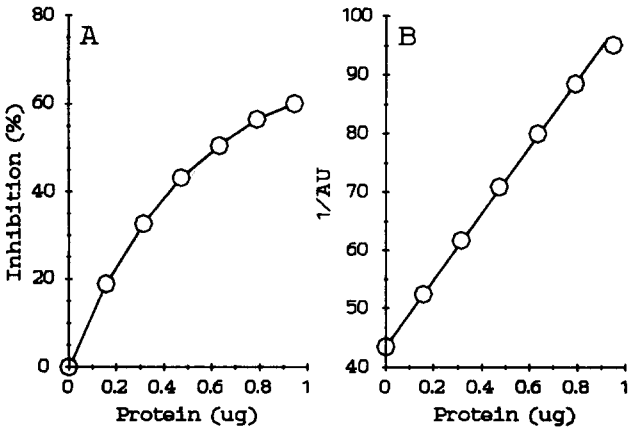

Fig. 6. (A) Effect of concentration of wheat extract protein on inhibition of 0.5 μg MFM amylase (preincubation volume = 200 μl 50 mM glycine pH 9.5 containing 0.5 mg/ml BSA). Values are means based on three wheat extracts with duplicates at each concentration. SE values are less than the width of data points. (B) Dixon plot of concentration of wheat extract against reciprocal velocity. K_i (0.79 μg) was estimated from the X intercept of a linear program analysis.

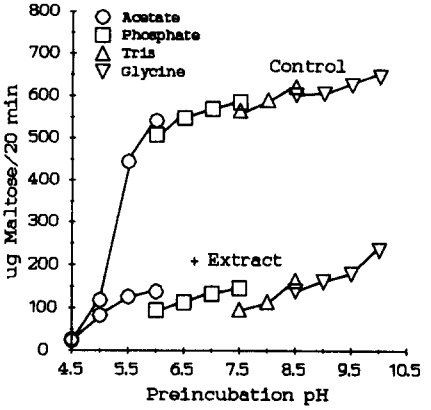

Fig. 8. Effect of preincubation pH on inhibition of MFM amylase by wheat extract. Residual amylase activity was assayed in 0.1 M glycine pH 9.0. Values are means of two separate tests. SE values are less than the width of data points.

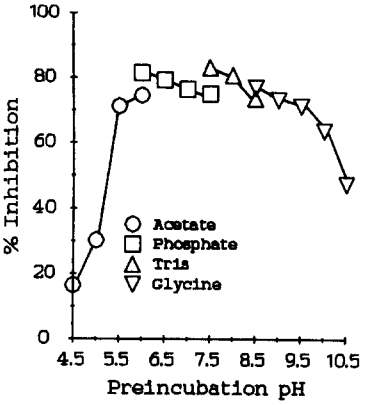

Fig. 9. Effect of pH on percentage inhibition of MFM amylase by wheat extract. Values are means of two separate tests. SE values are less than the width of data points.

DISCUSSION

Partially-purified MFM amylase is composed of two major and two minor isozymes arranged in pairs with slightly different molecular weights, about 50–51 kDa for the slower migrating pair and 56 kDa for the faster migrating pair. Each minor isozyme has the same molecular weight as its co-migrating major component but is slightly more anodic. These minor isozymes may be false isozymes and may result from deamidation of the major amylase proteins (Robinson *et al.*, 1970). Deamidation, which results in a slightly more anodic protein or peptide, is favored under alkaline conditions (pH 8–9) (Kauffman *et al.*, 1970; Keller *et al.*, 1971). Since these same isozyme patterns are observed in individual MFM larval midguts, the deamidation reaction could occur within the alkaline midguts of this species rather than during purification.

Optimum amylase activity between pH 9.0–9.5 for the granivorous *A. kuehniella* is similar to reported values for phytophagous larvae of *Bombyx mori* L. (pH 9.2) (Kanekatsu, 1973), *Spodoptera littoralis* Bois. (pH 9.5) (Ishaaya *et al.*, 1971), *Mamestra brassicae* L. (pH 9.5) (Kusano and Tanabe, 1986), and *Erinnyis ello* (pH 9.8) (Santos *et al.*, 1983). Preliminary studies with amylase of the rice moth, *Corcyra cephalonica* (Stainton), indicated a pH activity profile identical to that of *A. kuehniella*.

Chloride ion had no significant effect on K_m, velocity, or pH optimum of MFM amylase. Generally, insect amylases resemble mammalian amylases and are activated by chloride. For example, chloride activation of amylase has been demonstrated in Coleoptera (Buonocore *et al.*, 1976; Baker and Woo, 1985), Diptera (Terra *et al.*, 1977), Hemiptera (Hori, 1971), and Orthoptera (Evans and Payne, 1964; Moore and Davis, 1985). However, there are exceptions. Amylase from the beetle, *Callosobruchus chinensis* (L.) was inhibited by chloride (Podoler and Applebaum, 1971) and amylase from the cabbage armyworm, *M. brassicae*, was only weakly activated by chloride (Kusano and Tanabe, 1986). Additional Lepidopteran species need to be examined to determine if lack of chloride activation is a general property of alkaline amylases.

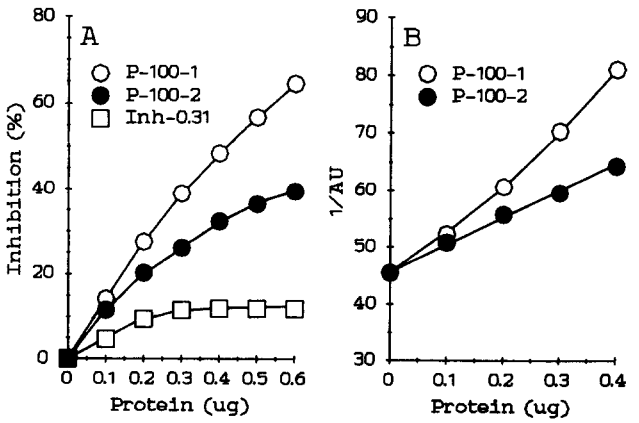

Fig. 10. (A) Effect of concentration of inhibitor-0.31 and partially-purified fractions P-100-1 and P-100-2 (from wheat) on inhibition of 0.5 μg MFM amylase at pH 9.5. (B) Dixon plot of reciprocal velocity against concentration of P-100-1 and P-100-2. Intercepts were obtained with a linear program analysis.

Purified amylases from *T. molitor* and *S. oryzae* are much more sensitive than MFM amylase to crude extracts of wheat as well as to purified amylase inhibitors (Buonocore *et al.*, 1980; Baker, 1987, 1988). Inhibitor-0.31 (mol. wt = *ca* 10 kDa; $K_i = 0.072$ nM) completely inhibits both amylase isozymes of *S. oryzae*. Inhibitor-0.12 (mol. wt = *ca* 10 kDa; $K_i = 11.4$ nM and 4.4 nM for the two isozymes) is less effective than inhibitor-0.31 but is still a potent inhibitor of *S. oryzae* amylase. In contrast, MFM amylase was completely insensitive to inhibitor-0.12 and was only slightly inhibited by inhibitor-0.31.

Partially-purified fractions P-100-1 and P-100-2 are more effective than inhibitors-0.31 and -0.12 against MFM amylase. A preliminary characterization of P-100-1 and P-100-2 has been undertaken (Baker and Woo, unpublished data). Three proteins with estimated molecular weights of 73 kDa were demonstrated in P-100-1 by the non-dissociating PAGE technique. P-100-2 was more complex and was composed of at least eight proteins inhibitory to one isoamylase of *S. oryzae*. Although MFM amylase is more sensitive to the high molecular weight inhibitors in P-100-1, the molecular basis for this increased sensitivity is unknown.

Complex formation between a purified inhibitor from wheat and amylase from *T. molitor* did not occur in alkaline conditions where the enzyme (pI = 4.0) and inhibitor (pI = 6.1) carried the same net charge (Buonocore *et al.*, 1980). It was postulated that ionic bonds formed at intermediate pH values (i.e. between pH 4 and 6.1) stabilized the complex formed between enzyme and inhibitor. Similarly, inhibition of *S. oryzae* amylase (pI = 3.7) by inhibitor 0.31 (pI = 6.1) was optimum between pH 4–5 and decreased rapidly in alkaline conditions above pH 7.5 (Baker, 1988). Stabilization of any complex formed between MFM amylase (pI = *ca* 4) and inhibitor-0.31 by ionic forces would be minimal at alkaline pH. This may partly explain the ineffectiveness of this inhibitor as well as inhibitor-0.12 against the alkaline amylase.

Acknowledgement—I thank S. M. Woo for excellent technical assistance during these studies.

REFERENCES

Baker J. E. (1982) Application of capillary thin-layer isoelectric focusing in polyacrylamide gel to the study of alkaline proteinases in stored-product insects. *Comp. Biochem. Physiol.* **71B,** 501–506.

Baker J. E. (1983) Properties of amylases from midguts of larvae of *Sitophilus zeamais* and *Sitophilus granarius*. *Insect Biochem.* **13,** 421–428.

Baker J. E. (1987) Purification of isoamylases from the rice weevil, *Sitophilus oryzae* (L.) (Coleoptera: Curculionidae), by high-performance liquid chromatography and their interaction with partially purified amylase inhibitors from wheat. *Insect Biochem.* **17,** 37–44.

Baker J. E. (1988) Purification of an α-amylase inhibitor from wheat, *Triticum aestivum*, and its interaction with amylases from the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Insect Biochem.* **18,** 107–116.

Baker J. E. (1989) Interaction of inhibitor-0.12 from wheat with two amylase isozymes from the rice weevil, *Sitophilus oryzae* (Coleoptera: Curculionidae). *Comp. Biochem. Physiol.* **92B,** 389–393.

Baker J. E. and Woo S. M. (1985) Purification, partial characterization, and postembryonic levels of amylases from *Sitophilus oryzae* and *Sitophilus granarius*. *Arch. Insect Biochem. Physiol.* **2,** 415–428.

Bryan J. K. (1977) Molecular weights of protein multimers from polyacrylamide gel electrophoresis. *Analyt. Biochem.* **78,** 513–519.

Buonocore V., Poerio E., Silano V. and Tomasi M. (1976) Physical and catalytic properties of α-amylase from *Tenebrio molitor* L. larvae. *Biochem. J.* **153,** 621–625.

Buonocore V., Petrucci T. and Silano V. (1977) Wheat protein inhibitors of α-amylase. *Phytochemistry* **16,** 811–820.

Buonocore V., Gramenzi F., Pace W., Petrucci R., Poerio E. and Silano V. (1980) Interaction of wheat monomeric and dimeric protein inhibitors with α-amylase from yellow mealworm (*Tenebrio molitor* L. larva). *Biochem. J.* **187,** 637–645.

Davis B. J. (1964) Disc electrophoresis—II. Method and application to human serum proteins. *Ann. N.Y. Acad. Sci.* **121,** 404–427.

Day M. F. and Waterhouse D. F. (1953) The mechanism of digestion. In *Insect Physiology* (Edited by Roeder K. D.) pp. 311–330, John Wiley & Sons, New York.

Evans W. A. L. and Payne D. W. (1964) Carbohydrases of the alimentary tract of the desert locust, *Schistocerca gregaria* Forsk. *J. Insect Physiol.* **10,** 657–674.

Hedrick J. L. and Smith A. J. (1968) Size and charge isomer separation and estimation of molecular weights of proteins by disc gel electrophoresis. *Archs Biochem. Biophys.* **126,** 155–164.

Hori K. (1971) Physiological conditions in the midgut in relation to starch digestion and the salivary amylase of the bug *Lygus disponsi*. *J. Insect Physiol.* **17,** 1153–1167.

Ishaaya I., Moore I. and Joseph D. (1971) Protease and amylase activity in larvae of the Egyptian cotton worm, *Spodoptera littoralis*. *J. Insect Physiol.* **17,** 945–953.

Kanekatsu R. (1973) Purification and some properties of amylase in the digestive juice of the silkworm larvae, *Bombyx mori*. *J. Sericult. Sci. Japan* **42,** 285–292.

Kauffman D. L., Zager N. I., Cohen E. and Keller P. J. (1970) The isozymes of human parotid amylase. *Archs Biochem. Biophys.* **137,** 325–339.

Keller P. J., Kauffman D. L., Allan B. J. and Williams B. L. (1971) Further studies on the structural differences between the isoenzymes of human parotid α-amylase. *Biochemistry* **10,** 4867–4874.

Kusano T. and Tanabe S. (1986) Enzymatic properties of the midgut amylase activity and its changes during development in the cabbage armyworm, *Mamestra brassicae* L. *Kontyu (Tokyo)* **54,** 12–24.

Lowry O. H., Rosebrough N. J., Farr A. L. and Randall R. J. (1951) Protein measurement with the Folin phenol reagent. *J. biol. Chem.* **193,** 265–275.

Loyter A. and Schramm M. (1962) The glycogen–amylase complex as a means of obtaining highly purified α-amylases. *Biochim. biophys. Acta* **65,** 200–206.

Moore K. C. and Davis G. R. F. (1985) Physical and catalytic properties of amylase from the alimentary canal of the migratory grasshopper, *Melanoplus sanguinipes* (Fab.). *Archs int. Physiol. Biochim.* **93,** 171–174.

Podoler H. and Applebaum S. W. (1971) The α-amylase of the beetle, *Callosobruchus chinensis*. *Biochem. J.* **121,** 321–325.

Powers J. R. and Culbertson J. D. (1982) *In vitro* effect of bean amylase inhibitor on insect amylases. *J. Food Protect.* **45,** 655–657.

Robinson A. B., McKerrow J. J. and Cary P. (1970) Controlled deamidation of peptides and proteins: an experimental hazard and a possible biological timer. *Proc. natn. Acad. Sci. USA* **66,** 753–757.

Santos C. D., Ferreira C. and Terra W. R. (1983) Consumption of food and spatial organization of digestion in the cassava hornworm, *Erinnyis ello*. *J. Insect Physiol.* **29**, 707–714.

Silano V., Furia M., Gianfreda L., Macri A., Palescandolo R., Rab A., Scardi V., Stella E. and Valfre F. (1975) Inhibition of amylases from different origins by albumins from the wheat kernel. *Biochem. biophys. Acta* **391**, 170–178.

Terra W. R., Ferreira C. and De Bianchi A. G. (1977) Action pattern, kinetical properties and electrophoretical studies of an alpha-amylase present in midgut homogenates from *Rhynchosciara americana* (Diptera) larvae. *Comp. Biochem. Physiol.* **56B**, 201–209.

Vittozzi L., De Angelis G. and Silano V. (1987) Solvent perturbation spectra of yellow mealworm α-amylase and of wheat protein inhibitors. *Int. J. Biochem.* **19**, 281–284.

Wiggleworth V. B. (1965) *The Principles of Insect Physiology*. Methuen, London.